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In vivo analysis of a fluorescent SUMO fusion in transgenic *Drosophila*

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Sumoylation, the covalent attachment of SUMO, a 90 amino acid peptide related to ubiquitin, is a major modulator of protein functions. Fluorescent SUMO protein fusions have been used in cell cultures to visualize SUMO in vivo but not in multicellular organisms. We generated a transgenic line of *Drosophila* expressing an mCherry-SUMO fusion. We analyzed its pattern in vivo in salivary gland nuclei expressing Venus-HP1 to recognize the different chromatin components (Chromocenter, chromosome IV). We compared it to SUMO immunostaining on squashed polytene chromosomes and observed similar patterns. In addition to the previously reported SUMO localizations (chromosome arms and chromocenter), we identify 2 intense binding sites: the fourth chromosome telomere and the DAPI-bright band in the region 81F.

Introduction

Among the various post-translational modifications known to target proteins, sumoylation has been increasingly described as a major modulator of protein functions. Sumoylation involves the covalent binding on particular lysines of a 90 amino acid polypeptide, SUMO. SUMO is structurally related to ubiquitin (SUMO means small ubiquitin like modifier).[1] However, in contrast to ubiquitination, which usually targets proteins to degradation, sumoylation acts as a major switch modifying protein-protein interactions, protein activity, or protein sub-cellular localization. For example, sumoylation of the transcription factor Sp3 allows the recruitment of the transcriptional repressors MEP-1, Mi-2, and Sfmbt, which bind SUMO.[2] Sumoylation of the chromatin regulator Su(Var)3–7 is required for its localization on the chromocenter.[3] Sumoylation of Mod(mdg4)-67.2 is required for its integration into insulator bodies.[4] Binding of SUMO requires E1 activating enzymes (SAE1 and SAE2) and E2 conjugating enzyme (Ubc9).[5,6] Distinct SUMO E3 ligases can also be involved, which gives substrate specificity to the reaction.[5,6] Sumoylation is reversible through the activity of SUMO proteases. This allows the system to be very dynamic. For example, the SUMO specific protease 2 SENP2 downregulates Polycomb silencing by de-sumoylating Pc2/CBX4, which reduces its recruitment to its targets *Gata4* and *Gata6* during heart development in mouse.[7]

Sumoylation is widely conserved in eukaryotes. In *Drosophila*, in contrast to vertebrates, there is only 1 gene encoding a SUMO homolog, *smt3*.[8] In this organism, more than a hundred proteins have been shown to be sumoylated.[9] Sub-cellular localization of SUMO in *Drosophila* cells is not uniform.[9] For example, in embryonic mitotic cells SUMO is localized on pericentromeric regions of condensed chromosomes.[9] This suggests that spatially restricted sumoylated targets account for the observed SUMO distribution. In order to follow SUMO in living *Drosophila* tissue, we generated a transgenic line expressing a fluorescent fusion of SUMO, mCherry-SUMO. We analyzed its pattern in vivo in salivary gland nuclei and observed previously unidentified SUMO binding sites. These sites are also observed for the endogenous SUMO on squashed salivary gland polytene chromosomes.

Results

In vivo observation of a SUMO fluorescent fusion

SUMO fluorescent fusions have already been used successfully in cell cultures,[10,11] which indicates that although the GFP is a little more than twice the size of SUMO (238 vs. 90 amino acids), it does not interfere too much with the conjugation of SUMO. GFP-SUMO was even shown to be able to replace SUMO in fission yeast.[12] Because the SUMO precursor undergoes maturation by cleavage of C-terminal

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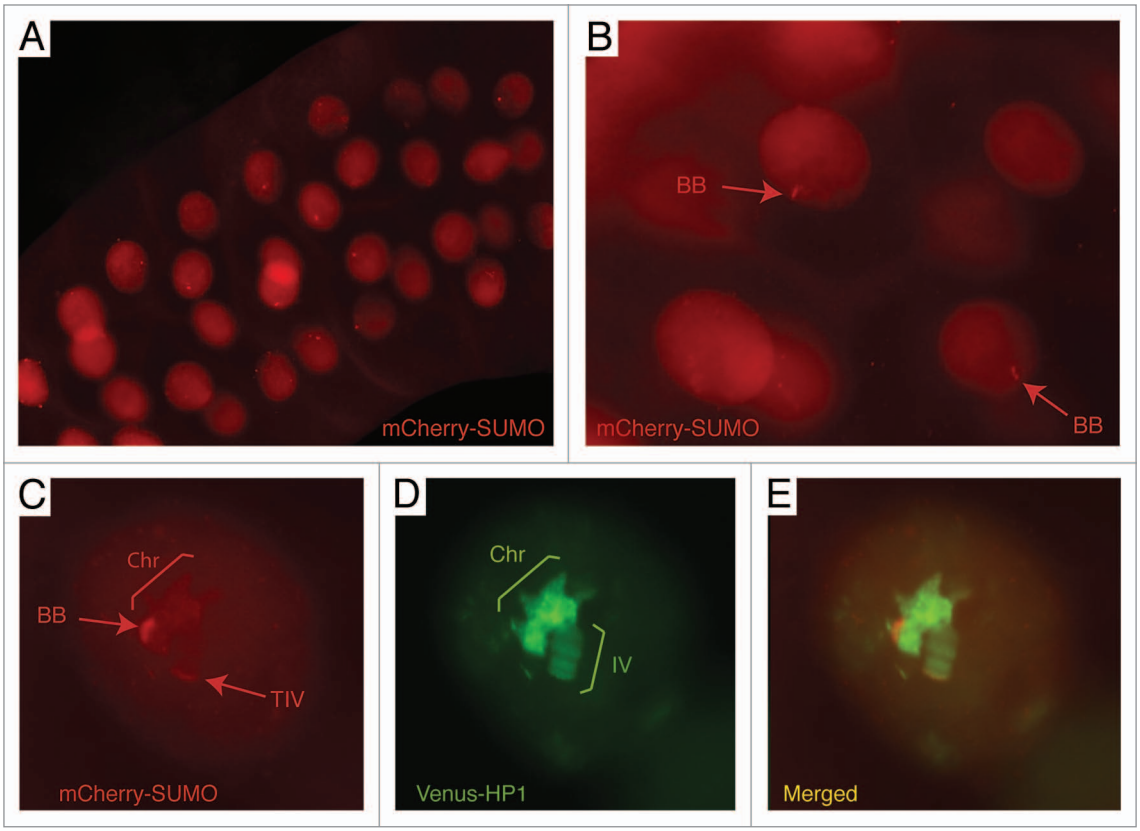

Figure 1. In vivo observation of mCherry-SUMO in salivary glands (**A and B**). BB: a bright band is observed in most nuclei. (**C–E**) In vivo observation of mCherry-SUMO (**C and E**; red) and Venus-HP1 (**D and E**; green) in a salivary gland nucleus. (**E**) Merged. BB: mCherry-SUMO bright band. TIV: SUMO staining on chromosome IV telomere. Chr: chromocenter. IV: chromosome IV.

residues,[6] the fluorescent tag must be fused to the N-terminal region. We generated a transgenic line to express a N-terminal mCherry-SUMO fusion in *Drosophila*. We chose the salivary glands to observe the pattern of our fluorescent fusion. Indeed, the polytene chromosomes of *Drosophila* salivary glands have been extensively used to visualize chromatin factors and the pattern of SUMO has been described briefly in this cell type.[13,14] We used the conditional UAS/Gal4 expression system[15] and chose the HeatShock:Gal4 driver whose leaky expression in the absence of heat shock allows a moderate expression in salivary glands.[16] We observed that the mCherry-SUMO fusion protein is mainly nuclear (**Fig. 1A and B**), like endogenous SUMO.[9] In most nuclei, a very bright band was observed in nuclear periphery (**Fig. 1B**, BB). A weaker band could be detected in proximity to this bright band at higher magnification. In order to recognize the major chromatin territories, we generated a transgenic line expressing a Venus-HP1 fusion. This allowed identifying the chromocenter and the chromosome IV. We co-expressed Venus-HP1 with mCherry-SUMO (**Fig. 1C–E**). Visualization of the chromocenter with Venus-HP1 shows that it is enriched in mCherry-SUMO (**Fig. 1C–E**). Furthermore, the bright mCherry-SUMO band was localized close to the chromocenter and the weaker mCherry-SUMO band maps to chromosome IV telomere (**Fig. 1C–E**).

SUMO pattern on squashed salivary gland polytene chromosomes

We were surprised to observe these 2 bright bands with mCherry-SUMO as they had not been reported previously on squashed polytene chromosomes.[13] We therefore analyzed precisely the endogenous SUMO binding pattern on squashed salivary gland polytene chromosomes, in wild type larvae, using a polyclonal antibody directed against *Drosophila* SUMO.[9] We observed that SUMO is visible in bands on chromosome arms and is enriched on the chromocenter (**Fig. 2**) as previously described.[13] The signal on the chromocenter is stronger that the signal on the arms. This latter signal is barely visible if the signal on the chromocenter is not saturated (**Fig. 2**). In addition, we observed 2 particularly strong binding sites: the telomere of the fourth chromosome and the DAPI bright band localized in the region 81F of the chromosome 3R, close to the chromocenter (**Fig. 2**). Remarkably, the intense staining on the DAPI bright band is not localized precisely on the DAPI signal but seems to envelop it. The pattern observed on squashed wild-type chromosomes with the anti SUMO antibody is therefore similar to the pattern observed with the mCherry-SUMO fusion in vivo in salivary gland nuclei of transgenic flies.

Discussion

In order to follow SUMO pattern in vivo in *Drosophila* tissues, we generated a transgenic line expressing a mCherry-SUMO

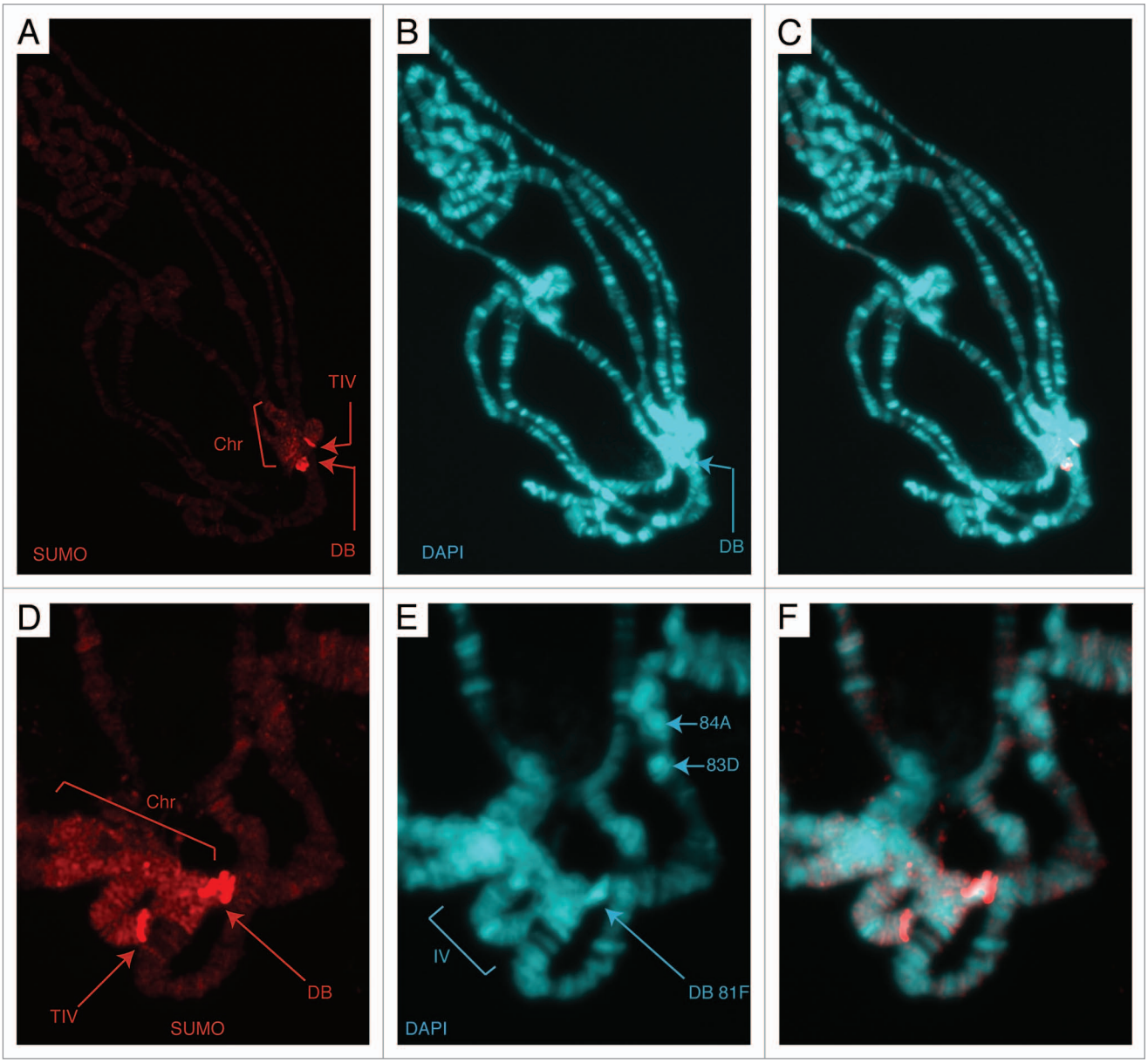

Figure 2. Immunostaining for SUMO (**A**, **C**, **D**, **and F**; red) on squashed salivary gland wild-type polytene chromosomes. DNA is stained with DAPI (blue). (**C and F)** Merged. DB: DAPI bright band. Chr: chromocenter. TIV: chromosome IV telomere. IV: chromosome IV. The SUMO signal was adjusted not to saturate on the DAPI bright band, the telomere of the fourth chromosome and the chromocenter, so it appears weak on chromosome arms.

fusion. The mCherry-SUMO fusion is nuclear like the endogenous SUMO.[9] The very low level of SUMO conjugates in the cytoplasm is supposed to be controlled by the SUMO protease Ulp1, localized to nuclear pores.[17] The pattern we observed in vivo in salivary glands with mCherry-SUMO is similar to the pattern we observed on squashed polytene chromosomes with an antibody against the endogenous SUMO. In addition to a staining on the chromocenter, we observed with both methods 2 intense binding sites not previously reported: the DAPI bright band in the region 81F close to the chromocenter and the telomere of the fourth chromosome. Thus, the pattern detected on squashed salivary gland polytene chromosomes of wild-type larvae corroborates with the pattern observed in vivo with the fluorescent fusion. The mCherry-SUMO fusion could therefore be useful for further studies. Indeed, SUMO fluorescent fusions have already been described in cell cultures[10,11] and in yeast,[12] but it is the first report, to our knowledge, of a fluorescent SUMO fusion expressed in a living metazoan. The signals on the DAPI bright band and on fourth chromosome telomere were not previously described on squashed polytene chromosomes with a SUMO antibody. These studies reported only signals on bands on chromosome arms and strong signal on the chromocenter.[13,14] Differences of fixation protocols or exposure time during image capture might explain these differences.

Material and Methods

Generation of transgenic lines expressing mCherry-SUMO and Venus-HP1

The coding sequences of SUMO (encoded by *smt3*: FBgn0264922) or HP1 (encoded by Su(var)205: Fbgn0003607) were amplified by PCR with primers containing restriction enzyme sites, cloned in p GEM-T Easy (Promega), and sequenced. They were then cut by the appropriate enzymes and inserted in the vector pUASTattB[18] in which the coding sequence of mCherry[19] or Venus[20] had been inserted before, as previously reported.[16] The fusion constructs were checked by sequencing. The constructs were integrated in the landing sites 58A (mCherry-SUMO) or 22A (Venus-HP1) on the second chromosome by injection of embryos with a source of integrase on the X chomosome.[18]

Fly culture and alleles

Flies were grown on standard corn-agar medium. We used standard balancer chromosomes to follow transgenes. In order to co-express Venus-HP1 and mCherry-SUMO, flies *UAS-mCherry-SUMO; HS-Gal4* were crossed to flies *UAS-Venus-HP1*.

Immunostaining on squashed polytene chromosomes

Larvae were dissected in Tyrode Buffer. Salivary glands were fixed for 30 s in 50µl of solution 2 (100mM NaCl, 2mM KCl, 2% Triton, 4% Paraformaldehyde, 10mM Phosphate buffer) and incubated then for 1 min 30 s in 50µl of solution 3 (45% acetic acid, 4% Paraformaldehyde). Salivary glands were then squashed. The quality of the chromosome spreads was assessed on a phase contrast microscope. The slides were then frozen in liquid nitrogen. Immunostainings were done using polyclonal rabbit anti SUMO antibody.[9] The chromosome spreads were incubated for 1 h with blocking solution (3% BSA, 0.2% NP40, 0.2% Tween 20, 10% non fat dry milk in PBS). They were incubated overnight at 4 °C in a humid chamber with 35µl primary antibodies diluted in blocking solution (anti-SUMO 1/500). They were then rinsed in PBS and washed twice 20 min in PBS, 300mM NaCl, 0.2% NP40, 0.2% Tween 20, and once 20 min in PBS, 400mM NaCl, 0.2% NP40, 0.2% Tween 20. They were rinsed in PBS and incubated for 1 h 30 min with goat anti rabbit secondary antibodies coupled to Alexa-555 in blocking solution. They were then rinsed in PBS and washed twice 20 min in PBS, 300mM NaCl, 0.2% NP40, 0.2% Tween 20 and once 20 min in PBS, 400mM NaCl, 0.2% NP40, 0.2% Tween 20. They were rinsed in PBS and mounted in Vectashield containing DAPI (Vector laboratories).

Fluorescence microscopy

Observations and image captures were made on an Axioplan microscope (Zeiss) with an Optronix camera and Magna-Fire software. Live salivary glands were observed in 0.7% NaCl.

The bright band in the nuclei of salivary gland expressing mCherry-SUMO was observed in 14 pictures.

The bright band and the telomere of the fourth chromosome were observed simultaneously on 4 salivary gland nuclei (from 3 independent pictures) from salivary glands expression mCherry-SUMO and Venus-HP1.

The SUMO staining on the fourth chromosome telomere and on the DAPI bright band were observed independantly on many chromosome squashes and simultaneously on 6 chromosome squashes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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